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DNA sequences of troglobitic nicoletiid insects support Sierra de El Abra and the Sierra de Guatemala as a single biogeographical area: Implications for *Astyanax*

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Abstract

The blind Mexican tetra fish, *Astyanax mexicanus*, has become the most influential model for research of cave adapted organisms. Many authors assume that the Sierra de Guatemala populations and the Sierra de El Abra populations are derived from two independent colonizations. This assumption arises in part from biogeography. The 100 m high, 100 m wide Servilleta Canyon of the Boquillas River separates both mountain ranges and is an apparent barrier for troglobite dispersion. *Anelpistina quinterensis* (Nicoletiidae, Zygentoma, Insecta) is one of the most troglomorphic nicoletiid silverfish insects ever described. 16S rRNA sequences support that this species migrated underground to reach both mountain ranges within less than 12,000 years. Furthermore, literature shows a plethora of aquatic and terrestrial cave restricted species that inhabit both mountain ranges. Thus, the Servilleta canyon has not been an effective biological barrier that prevented underground migration of troglobites between the Sierra de Guatemala and the Sierra de El Abra. The Boquillas River has changed its course throughout time. Caves that in the past connected the two Sierras were only recently geologically truncated by the erosion of the new river course. It is likely that, with the geological changes of the area and throughout the 2-8 million years of evolutionary history of cave *Astyanax,* there have been opportunities to migrate across the Servilleta canyon.

Keywords

Anelpistina quinterensis, Neonicoletia, Cubacubaninae, Nicoletiidae, Zygentoma, Insecta, Thysanura, Silverfish, *Astyanax,* blind tetra, Characidae, Sierra de El Abra, Sierra de Guatemala,16S rRNA, Molecular clock, Colonization

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Introduction

In recent years, the blind Mexican tetra fish *Astyanax mexicanus* (De Filippi, 1853) has become the most influential model for genomic and evolutionary research of cave adapted organisms. Regrettably, there is great confusion regarding the origin of the 29 populations that inhabit the Sierra de El Abra, Sierra de Guatemala, and Micos mountain ranges in Northeastern Mexico and, also, if the populations derived from a single or from multiple colonizations. A plethora of publications has accumulated over time with terms such as phylogenetically old/new populations, lineages A/B, phylogenetically old/new clusters, and old/new epigean stocks, with individual cave fish populations having been assigned contradictorily to one or to another set (see for example figure 1 in Gross 2012).

Many current authors embrace the hypothesis that Sierra de Guatemala populations derived from a new epigean stock and that Sierra de El Abra populations derived from an old stock. This is complicated by some El Abra populations, such as the Pachón cave population, having subsequently hybridized with the new stock (Bradic et al. 2012). It is seldom assumed that the Guatemala populations could also have an old stock origin which has then been obscured by extensive hybridization with the new stock, and much less that populations from both mountain ranges could have a single underground cave adapted ancestor. One reason derives from biogeography and an apparent barrier between the two mountain ranges. The Cañon de la Servilleta (Napkin canyon) of the River Boquillas separates both mountain ranges (Figure 1). Reddell (1981) subdivided the two mountain ranges into separate biogeographical areas and authors working with *Astyanax*, such as Gross (2012), have assign populations to either region based on geography, regardless of there being no genetic studies (ex. Jineo, Bee and Vasquez caves). Intrinsically, it has been assumed that this 100 m high, 100 m wide canyon has been a biological barrier that prevented underground migration of troglobites between the two karstic areas, and therefore colonization had to occur independently on both mountain ranges.

The purpose of this paper is not to resolve if troglobitic *Astyanax* derived from single or multiple origins. What we will address is if the Servilleta canyon has been an effective barrier for migration of troglobites in general, and thus if the Sierra de Guatemala and the Sierra de El Abra should be considered two separate cave biogeographic areas. For this, the DNA sequences of troglobitic nicoletiid insects (Zygentoma, also known as silverfish or Thysanura) of genus *Anelpistina* from populations inhabiting both Sierras were analyzed and a phylogeny was obtained. Our results will help to establish if these troglobites are a single or multiple species, and thus support if they are the product of a single colonization followed by underground migrations, or derived from multiple colonizations.

Anelpistina quinterensis (= *Neonicoletia quinterensis* Paclt, 1979) is a rather large troglobite (8.5 cm long, antennae and terminal filaments or caudal appendages included), which was first described from Grutas de Quintero, in Sierra de El Abra. When re-describing the species, Espinasa et al. (2007) reported its presence in Pachón

Figure 1. The Cañon de la Servilleta of the River Boquillas separates the contiguous Sierra de Guatemala, to the north, from the Sierra de El Abra, in the south. Limestone is restricted to the green forested hills. This study tested if this 100 m high, 100 m wide canyon was an effective biological barrier that prevented underground migration of troglobites between the two karstic areas.

and Yerbaniz Caves, also within the Sierra de El Abra. They mentioned that "It is likely that *Anelpistina quinterensis* is restricted to the caves of Sierra de El Abra". With their highly elongated legs, antennae (almost thrice as long as the body), and caudal appendages (twice as long as the body), it is one of the most troglomorphic nicoletiids ever described (Figure 2). They can be found walking on mud banks and they are probably restricted to highly humid environments. In caves where they are abundant, they are never found near the entrance or in drier passages. As a very highly adapted troglobite, it is very unlikely that it can survive on the surface and its habitat must be restricted to underground passages. Its range probably reflects connectivity within a karstic area throughout geologic times and during the evolutionary history of the species.

Methods

Three caves near the town of Gómez Farías in the Sierra de Guatemala are inhabited by *Anelpistina* populations whose taxonomic identity has not previously been defined: Sótano de los Mangos, Sótano del Plan, and Sótano de Jineo. Two specimens per cave were studied and their DNA extracted. For this study, the 16S rRNA sequences of two *A. quinterensis* from Grutas de Quintero were already available in GeneBank (DQ280127.1). Also from Sierra de El Abra, two new specimens of *A. quinterensis* from Sabinos cave were obtained (3/20/13). For reference, the caves of Sabinos, Pachón and Sótano de Jineo can

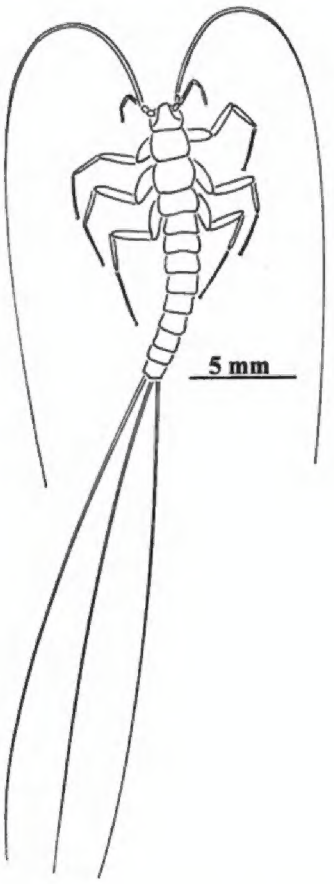

Figure 2. *Anelpistina quinterensis* is one of the most troglomorphic described species of nicoletiids. This relatively large eyeless insect is albino and has extremely elongated appendages. Its habitat is restricted to very humid portions of the caves such as mud banks. It is doubtful that it can survive in an epigean environment. Its habitat probably reflects connectivity within a karstic area throughout geologic times and during the evolutionary history of the species.

be found in figure 1 of Gross (2012) and described in Mitchell et al. (1977). Sótano de los Mangos and Sótano del Plan are in the neighboring area of Sótano de Jineo. Grutas de Quintero is near Pachón cave, but on the eastern side of the Sierra de El Abra.

Genomic DNA was extracted using Qiagen's DNEasy® Tissue Kit by digesting a leg in lysis buffer. Amplification and sequencing of the 16S rRNA fragment followed standard protocols and primers for the 16S rRNA fragment used in the past for nicoletiids (Espinasa and Giribet 2009). Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software SequencherTM 3.0. External primers were excluded from the analyses. Sequences from the new *Anelpistina* populations (GenBank# KF917530-KF917534) and sequences of all other nicoletiid species available in GeneBank were aligned and neighbor joining analysis was performed using ClustalW2.

Results

The 16S rRNA fragment from the six specimens from the three caves of Sierra de Guatemala was identical and 499 bp long. The two Sabinos Cave specimens differed among themselves by two bp (0.4%) and were 498 bp. The Quintero specimens were

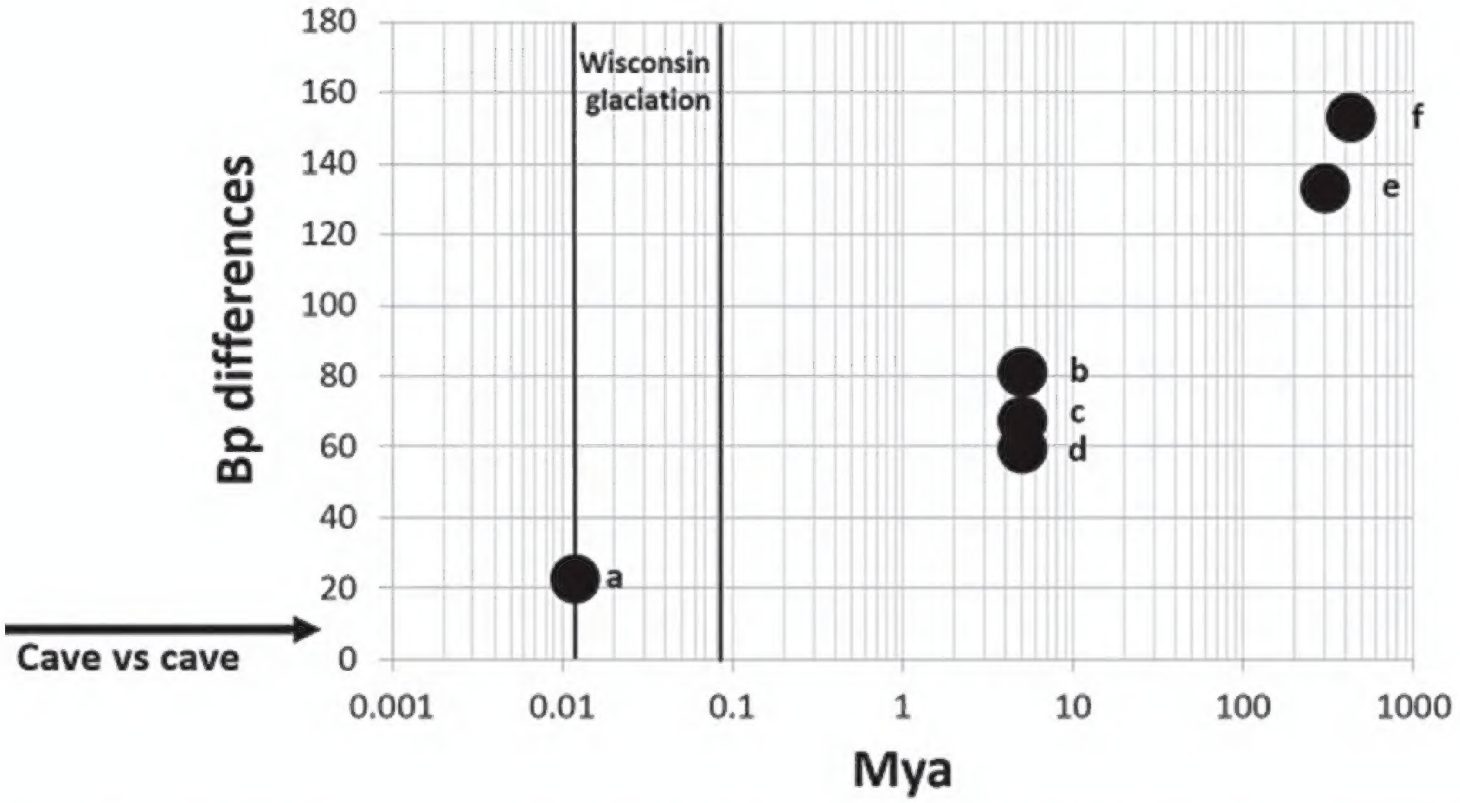

Figure 3. Base pair differences versus estimates of divergence in nicoletiids. Base pair differences in the 16S rRNA fragment is plotted against estimates of divergence times millions of years ago (Mya). Molecular clock calibrating points were extracted from: **a** populations of *Anelpistina musticensis* that got separated into different islands when the sea level rose after glacial times 12,000 years ago (Espinasa et al. 2011) **b** and **c** species of *Prosthecina* and **d** species of *Anelpistina* from Baja California that got separated from the mainland species when the Gulf of Cortes formed 5 mya (Espinasa et al. 2009) **e** time when nicoletiids arose from a common ancestor with Lepismatids 302 mya (Regier et al. 2010), and **f** time when insects arose from a common ancestor with anostraca in the Silurian-Ordovician boundary 427 mya (Gaunt and Miles 2002). The lower arrow indicates the 11–12 bp differences between the Sierra de Guatemala and the Sierra de El Abra *Anelpistina* populations. Such sequence difference is consistent with a common origin very recently, less than 12,000 years ago, and therefore after the environmental disturbances of the ice age.

identical and 498 bp. Within the Sierra de El Abra, specimens from Quintero and Sabinos differed among each other by 5 bp (1%). The Sierra de Guatemala specimens differed from the Quintero specimens by 11 bp (2.2%) and from the Sabinos specimens by 12 bp (2.4%). The neighbor joining analysis showed all to be monophyletic and very distant from any other nicoletiid insect that has had their 16S sequenced, including surface specimens from the neighboring areas.

A comparison of the DNA differences among the *Anelpistina* of Sierra de El Abra and Sierra de Guatemala was made against other nicoletiid species with dated speciation events (Figure 3). When the molecular clock was originally calibrated for nicoletiids (Espinasa et al. 2011), one point in particular was used for the end of the ice age. During glacial times when the sea level was lower, the islands of Mustique and Union Island (Grenadine islands in the Caribbean) formed a single land mass. The nicoletiid populations of *Anelpistina musticensis* separated and were isolated 12,000 years ago when the sea levels started to rise. These isolated populations now have 16S rRNA fragments that differ by 21 bp (Espinasa et al. 2011). The 11-12 bp difference

between the Sierra de El Abra and Sierra de Guatemala populations implies that these cave populations shared a common ancestor fairly recently, about 5,000 years ago, and certainly less than 12,000 years ago when the ice age ended. Such a recent origin supports that the *Anelpistina* populations belong within the same species. This is also in agreement with data from the 16S rRNA fragment sequences of nicoletiid species across the subfamily Cubacubaninae (Espinasa and Giribet 2009), where the 11-12 bp difference is within the range of 22 different populations that belong to the same species. Furthermore, morphologic analyses failed to find any discriminative character between the Guatemala and the El Abra populations. It is therefore supported that all populations from both Sierras belong to *A. quinterensis*.

Discussion

Our results support that troglobitic *A. quinterensis* from both Sierras had a common ancestor less than 12,000 years ago. We believe that this fairly recent common ancestor of the *A. quinterensis* population was a cave adapted organism which, through systems of caves and microcaves, migrated underground to reach and establish the current cave populations. As mentioned above, *A. quinterensis* may not survive on the surface and is one of the most troglomorphic nicoletiid insects. With such a recent common ancestor, it is unlikely that a surface ancestor would have had enough evolutionary time to independently colonize the caves of both mountain ranges, and then convergently develop such an advanced degree of troglomorphy. It would also be extremely unlikely that this independent evolution would yield indistinguishable morphologies in the two derived populations. Finally, since this surface ancestor would have been present long after the disturbances of the ice age had ended and, therefore, when environmental conditions have remained relatively stable, it would be expected that the surface species would still be present. Search for nicoletiids on the surface has successfully resulted in collecting other species, but never a surface specimen of *A. quinterensis.* In conclusion, it appears that *A. quinterensis* has been able to migrate between the two sierras and, therefore, the Servilleta canyon has not been an effective barrier to its underground dispersal.

Anelpistina quinterensis is not alone in having been able to disperse between both mountain ranges. There are at least four aquatic troglobites shared between the Sierra de El Abra and the Sierra de Guatemala; "the entocytherid ostracod *Sphaeromicola cirolanae* Rioja, the cirolanid isopods *Speocirolana bolivari* (Rioja) and *S. pelaezi* (Bolivar), and the mysid *Spelaeomysis quinterensis* (Villalobos)" (Reddell 1981). At least six species of terrestrial troglobites are also found in both Sierras; "the squamiferid isopod *Spherarmadillo cavernicola* Mulaik, the trichoniscid isopod *Brackenridgia bridgesi* (Van Name), the amblypygid *Paraphrynus baeops* Mullinex, the opilionid *Hoplobunus boneti* (Goodnight and Goodnight), the centipede *Newportia sabina* Chamberlin, and the collembolan *Pseudosinella petrustrinatii* Christiansen" (Reddell 1981). As can be seen from this certainly incomplete list, there are many instances of troglobites inhabiting both areas. This plethora of shared troglobites indicates that in the evolution of cave organ-

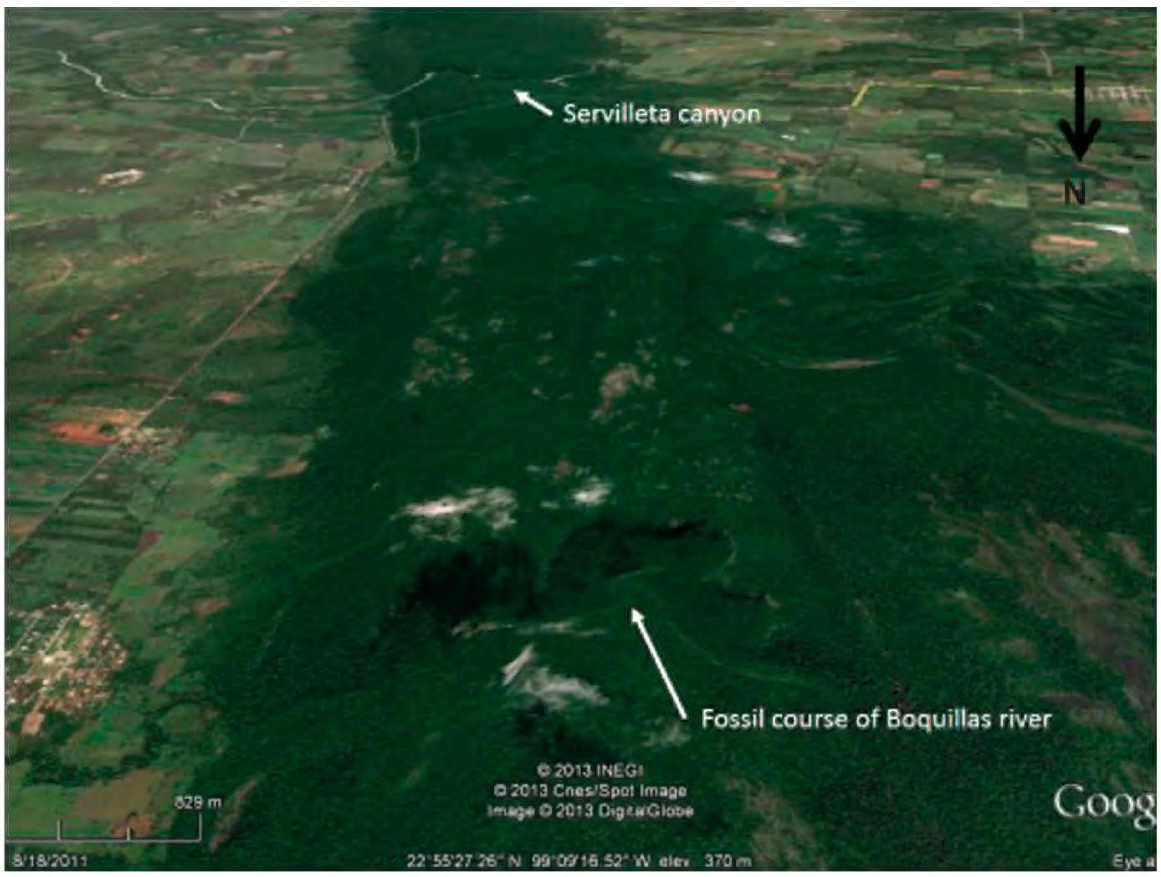

Figure 4. The Boquillas River has changed its course throughout time. The Boquillas River currently separates the karstic areas of Sierra de Guatemala from the Sierra the El Abra. In the upper part of the figure, the Boquillas River is seen crossing the sierras through the Servilleta canyon. On the bottom part of the figure, a fossil canyon indicates the river's ancient course. Caves that in the past connected the Sierra de El Abra in the south to the Sierra de Guatemala in the north were only recently geologically truncated by the erosion of the new river course. Limestone is restricted to the green forested hills.

isms, the Servilleta canyon has not been an effective biological barrier that prevented underground migration of troglobites between the Sierra de Guatemala and the Sierra de El Abra. Both karstic areas can therefore be considered a single biogeographical area.

Regarding its geologic history, Sierra de El Abra has been "emerging" as limestone is exposed by erosion, following the progressive lowering of the base level to the current elevation of the present coastal plain. Throughout this process, the river Boquillas, which currently divides Sierra de El Abra and Sierra de Guatemala, has vastly changed its course. As can be seen in Figure 4, there are the remains of a fossil river course further north of its current path. The Servilleta canyon was formed in relatively recent geological times when the Boquillas River changed its course to a more southern location and started cutting through the karstic layers. Exploration of the Servilleta canyon has revealed the presence of caves on one side of the canyon, and exactly on the other side of the canyon, with the same angle, complementary caves. This implies that there were caves connecting both mountain ranges which have recently been cut by the erosion of the Boquillas River. Biological dispersal of troglobites could have used these ancient caves. They could also use the connecting cavities that must exist below the current river level that have yet to be eroded by the Boquillas River. Alternatively, somehow they may have managed to survive the minor 100m "jump" between caves on either side of the canyon.

Conclusion

Regardless of the means used by troglobites to successfully migrate between the two mountain ranges, the main conclusion of this work is that the Servilleta canyon does not appear to be an effective biological barrier between the Sierra de Guatemala and the Sierra de El Abra. Troglobites of sizes comparable to the blind *Astyanax*, both aquatic and terrestrial, are found in both Sierras. *Astyanax* colonized the cave environment 2–8 million years ago (Gross 2012). Since nicoletiids have been able to migrate in between southern El Abra and the Sierra de Guatemala in less than the last 12,000 years, it is likely that, with the geological changes of the area throughout the evolutionary history of *Astyanax,* there have been opportunities to migrate across the current Servilleta canyon.

Undoubtedly the *Astyanax* populations of Sierra de El Abra and Sierra de Guatemala have been sufficiently isolated from each other so as to have, to a certain extent, independent evolutionary histories. This is reflected by microsatellite markers (Bradic et al. 2012) and the independent and parallel evolution of multiple troglomorphic characters such as albinism (Protas et al. 2006), brown phenotype (Gross et al. 2009), and the genetic basis of eye regression (Borowsky 2008), to give some examples. But as mentioned before, there is the possibility that the Guatemala populations may also have had an origin from the same old stock as the El Abra populations, but the genetic evidence has been obscured by extensive hybridization with the new stock.

Initial sequencing of mitochondrial DNA placed the Sierra de El Abra Pachón population within the new stock. Only subsequent studies showed its old stock origin having been obscured by hybridization. Some genetic markers also support that Sierra de Guatemala populations may as well have an old stock origin, but more intensely obscured by hybridization. For example, while most microsatellite markers (Bradic et al. 2012) of the Guatemala populations are shared with surface specimens, there is one allele (a6 f1-256) only present in the Sierra de Guatemala and Sierra de El Abra caves, but absent in surface populations. If the two sierras had actually been in separate biogeographic areas and the Servilleta canyon had been an effective barrier, the different *Astyanax* populations would undoubtedly be the result of independent colonization. Recognizing that there is no effective barrier for troglobite migration between the two areas, reduces this certainty. Genomic studies will resolve if a fraction of the genome of the cave fish from both sierras is shared at the exclusion of all surface populations.

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